Audio-Frequency NMR in a Nutating Frame. Application to the Assignment of Phenylalanine Residues in Isotopically Enriched Proteins

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Abstract: The effect of chemical shift can be removed to high order by quantizing nuclear spins along a strong radio-frequency (RF) field and applying a second audio-frequency magnetic field, orthogonal to the direction of the effective RF spin lock field. By adjusting the audio-modulation frequency of this second magnetic field to the rate at which spins nutate about the RF field, a resonance condition in the spin-locked frame is established which, to second order, is independent of the resonance offset of the homonuclear spins. The phase and amplitude of the audio-frequency field are under operator control and make it possible to conduct complex pulse sequences in the nutating frame. The approach is demonstrated for transferring in a single step magnetization from phenylalanine $^{13}C^{\beta}$ to all aromatic ring carbons in a sample of uniformly ^{13}C-enriched calmodulin. A three-dimensional version of the experiment yields complete resonance assignments for the structurally informative aromatic Phe signals, including all $^{13}C^{\zeta}$ and $^{1}H^{\zeta}$ resonances.

Rapid advances in protein NMR over the last decade have been fueled to a large extent by advances in multidimensional NMR methodology which have made it possible to establish unambiguous resonance assignments and to measure $^{1}H-^{1}H$ distances. Most of these modern experiments rely on the use of ^{13}C and ^{15}N enrichment of the protein in order to disperse its NMR spectrum in up to four orthogonal frequency dimensions.[1,2] Resonance assignments in such isotopically enriched proteins are commonly obtained by a combination of heteronuclear J correlation techniques, including HCCH-TOCSY and HCCH-COSY.[3,4] These standard experiments fail, however, for assigning the aromatic ring resonances of phenylalanine residues. Phe residues are almost invariably located in the hydrophobic interior of proteins and therefore are important for determining the solution structure of a protein at high accuracy. Resonance assignment of the Phe-H^{ζ} protons is particularly important as their position is not affected by aromatic ring flipping and they frequently exhibit large numbers of structurally-important long-range NOE constraints. Moreover, H^{ζ} is located at a fixed distance from the protein backbone and, provided the χ_1 side chain torsion angle can be determined, the position of H^{ζ} relative to the backbone is known. An ingenious scheme for transferring ^{13}C magnetization from C^{β} into the aromatic ring[5] by COSY type transfer steps frequently fails for Phe residues because the ^{13}C spectrum of the aromatic ring carbons is commonly subject to severe non-first-order effects, caused by the large size of the aromatic $^{1}J_{CC}$ couplings (*ca.* 55 Hz) and small differences in their ^{13}C shifts. These strong coupling effects, together with an unfavorable topology of the six-membered ring spin system, in practice therefore make it impossible to use such COSY-based transfer schemes for assignment of Phe-H^{ζ} resonances.

The strong coupling between the aromatic carbons makes it attractive to use isotropic mixing type experiments, which are aimed at removing the effects of ^{13}C chemical shift differences altogether. However, the difference between Phe-C^{β} and -C^{γ} chemical shifts can exceed 100 ppm and commonly used isotropic mixing schemes are not very efficient over such a wide bandwidth. Here we describe a new type of NMR experiment which can effectively generate cross polarization over such a bandwidth. In contrast to regular NMR experiments, where spins are quantized by the static B_0 field and subjected to radio-frequency (RF) pulses, in the new experiment the spins are quantized along a RF field, orthogonal to the static magnetic field. A series of audio-frequency pulses in the corresponding nutating frame is then used to induce transitions between the spin-locked frame eigenstates. The audio-frequency pulses may be applied either by modulating the static magnetic field at a frequency which is resonant in the nutating frame or by appropriate modulation of the applied RF field. In either case, the method utilizes *a*udio-*m*odulated *n*utation for *e*nhanced *s*pin *i*nter*a*ction between aromatic ring carbons and side chain $^{13}C^{\beta}$ spins, and will henceforth be referred to as AMNESIA. It may be considered an extension of an experiment for rotary saturation in solids[6] or early amplitude/frequency-modulated double resonance experiments in liquids[7] and it also has close similarities to rotating frame magnetic resonance imaging.[8] The principal difference is that in our implementation of the AMNESIA scheme we actually apply a relatively complex audio-frequency pulse sequence, rather than a continuous wave audio-frequency irradiation.

Theory

The principle of nutating frame spectroscopy is most easily understood for a one-spin system that is observed in a frame

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rotating about the z axis of the static magnetic field at an angular frequency, Ω. The regular RF coil of the spectrometer makes it possible to apply magnetic fields which rotate (neglecting counter-rotating components) perpendicularly to the static magnetic field with arbitrary phase and amplitude. In particular, on modern NMR spectrometers, the RF field can easily be modulated in such a way that a field oscillating at frequency ω_m is superimposed perpendicularly onto the normal "constant" RF field, e.g. such that the one-spin Hamiltonian takes the following form in the rotating frame:

$$\mathcal{H}(t) = \delta I_z + \omega_1 I_x + 2\alpha\omega_1 I_y \cos(\omega_m t) \quad (1)$$

where $I_{x,y,z}$ refer to the spin angular momentum operators in the x, y, and z dimensions, δ is the difference between the Larmor frequency of the spin I and Ω, $\omega_1 = \gamma B_1$ is the overall amplitude of the RF field, α is the amplitude of the modulation, and ω_m is its frequency. For $\alpha \ll 1$, eq 1 corresponds to a phase modulation, which is equivalent to a frequency modulation, of the applied RF field.[7] The term $2\alpha\omega_1 I_y \cos(\omega_m t)$, constitutes a linearly modulated field which can be decomposed into two components, rotating at frequencies $\pm\omega_m$ around the effective field formed by the terms δI_z and $\omega_1 I_x$. In practice the field strength ($\omega_a = \alpha\omega_1$) of those components will lie in the audio range. Neglecting the counter-rotating term, the audio field becomes static in a coordinate system rotating at frequency ω_m around the effective field.

The above situation is therefore exactly analogous to the conventional RF generated in the laboratory frame by sending a $\cos(\Omega t)$-modulated current through the RF coil. In the present case, the modulation of the field is done in the rotating frame. In conventional NMR, the effect of spin locking by a strong RF field reduces differences in the Zeeman terms (i.e., chemical shifts) in the rotating frame. As shown below, spin-locking in the doubly rotating frame can be used to reduce differences of the analogous effective field terms present in the singly rotating frame.

In eq 1, the term $2\alpha\omega_1 I_y \cos(\omega_m t)$ generates the audio field along the y-axis of the rotating frame. As mentioned above, this audio-field can be considered as the sum of two audio fields, each of amplitude $\alpha\omega_1$, rotating in opposite directions in the yz plane (in the limit where $\delta \to 0$). The assumption, commonly used in conventional NMR, that the effect of the so-called counter-rotating component may be safely neglected is no longer valid in the nutating frame where the ratio of the audio field over the effective field is much higher than the corresponding ratio ω_1/Ω in regular NMR experiments. In the present case, the counter-rotating audio-field component can be eliminated by the application of an additional sine modulation of the static z-field with frequency ω_m and amplitude $2\alpha\omega_1/\gamma$. As argued below, there is no need to apply such a modulation of the static field as the same effect can be accomplished without the need for extra hardware.

For small values of α, $\omega_m \approx \omega_1$, and in the limit $\delta \ll \omega_1$, such a circularly polarized field can also be obtained when transforming into an appropriate "wobbling frame", using the unitary transformation $U = \exp(iI_z\alpha \cos(\omega_m t))$ which transforms the Hamiltonian of eq 1 into

$$\mathcal{H}'(t) = \delta I_{z'} + \omega_1 I_{x'} + \alpha\omega_m I_{z'} \sin(\omega_m t) + \alpha\omega_1 I_{y'} \cos(\omega_m t) \quad (2)$$

where the primes refer to the spin operators in the wobbling frame, and $I_z = I_{z'}$. As is seen from eq 2, the audio field is circularly polarized in this wobbling frame. The effect of the counter-rotating component, present in the regular rotating frame, is now shifted to the transform of the density matrix from the rotating frame to the wobbling frame and the analogous transform from the wobbling to the rotating frame.

At the expense of a slightly more complex modulation function for the RF field, circularly polarized irradiation in the wobbling frame can be generated independent of the magnitude of α. In this case, the RF field needs to be modulated to yield the following Hamiltonian in the regular rotating frame:

$$\mathcal{H}_1(t) = \delta I_z + \omega_1[I_x(\cos\beta - \beta \sin\beta) + I_y(\sin\beta + \beta \cos\beta)] \quad (3)$$

where β defines the modulation function as $\beta = \alpha \cos(\omega_m t + \phi)$, with α being the amplitude of the modulation in radians, ω_m its frequency, and ϕ an arbitrary initial phase. Note that for $\alpha \to 0$ and $\phi = 0$, eq 3 reduces to eq 1. By applying the unitary transformation $[U_1 = \exp(iI_z\beta)]$, the Hamiltonian $\mathcal{H}_1$ of eq 3 is transformed to the wobbling frame, yielding:

$$\mathcal{H}_2(t) = \delta I_{z'} + \omega_1 I_{x'} + \alpha\omega_m I_{z'} \sin(\omega_m t + \phi) + \alpha\omega_1 I_{y'} \cos(\omega_m t + \phi) \quad (4)$$

where the primes refer to the spin operators in the wobbling frame, and $I_z = I_{z'}$. The effect of the "wobbling frame" transformation on the initial and final states of the density matrix is merely a rotation around the z-axis by an angle $\alpha \cos(\omega_m t + \phi)$. For $\phi = 90°$ or 270°, the two frames coincide for $t = 0$, and when a windowless series of such pulses is applied no cumulative error results from the forth-and-back transforms between the regular rotating frame and the wobbling frame, provided that the audio-pulse duration corresponds to an integer multiple of π/ω_m. As is seen from eq 4, in the case where $\delta = 0$ and $\omega_m = \omega_1$, $\mathcal{H}_2$ has the same form as the Hamiltonian of a static Zeeman field along the x-axis of strength ω_1 and a purely circular field of strength ω_a which rotates at frequency ω_m around it.

In order to solve the dynamics of the system described by $\mathcal{H}_2(t)$, the coordinate system is rotated $[U_2 = \exp(iI_{y'}\theta)]$ around the y'-axis by an angle θ ($\tan\theta = \omega_1/\delta$) such that the new z'' axis is in the direction of the effective static field of amplitude $\omega_e = (\omega_1^2 + \delta^2)^{1/2}$.[9] A third transformation $[U_3 = \exp(iI_{z''}\omega_m t)]$ translates the Hamiltonian to a frame that is rotating with frequency ω_m around this effective field axis. We refer to this frame as the doubly rotating or nutating frame. In this frame, the Hamiltonian is then given by

$$\mathcal{H}_3(t) = [\omega_e - \omega_m - (\alpha\omega_m\delta/\omega_e) \sin(\omega_m t + \phi)]I_{z'''} - \omega_a/2\{(1 + \omega_m/\omega_e))[I_{y'''} \cos\phi - I_{x'''} \sin\phi] + (1 - (\omega_m/\omega_e))[I_{y'''} \cos(-2\omega_m t - \phi) - I_{x'''} \sin(-2\omega_m t - \phi)]\} \quad (5)$$

There is no general analytical solution to the Schroedinger equation for a periodic time-dependent Hamiltonian of the form of $\mathcal{H}_3$. However, if we limit ourselves to stroboscopic observations of the system at time points $2n\pi/\omega_m$ ($n = 0, 1, 2, \ldots$), $\mathcal{H}_3(t)$ can be replaced by a fast converging series of static Floquet Hamiltonians in the Schroedinger equation.[10] It is straightforward to carry this expansion to high orders. The first-order term which corresponds to the average Hamiltonian is given by

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$$\mathcal{H}^{F,I} = (\omega_e - \omega_m)I_z - (1/2)\omega_a(1 + \omega_m/\omega_e)[I_y \cos\phi - I_x \sin\phi] \quad (6)$$

where, for convenience, the triple primes have been omitted. $\mathcal{H}^{F,I}$ therefore consists of a reduced Zeeman field ($\omega_e - \omega_m$) along the new z-axis and an audio field perpendicular to it. The strength of the audio field is $\omega_a(1+ \omega_m/\omega_e)/2$ and its phase is $\phi - 90°$, both of which are completely under operator control and can be used to design pulse sequences in the nutating frame.

In a conventional spin-locking experiment, the difference of the effective fields that two spins (resonance frequencies δ_1 and δ_2) experience is reduced as compared to the normal Zeeman terms $[(\omega_1^2 + \delta_1^2)^{1/2} - (\omega_1^2 + \delta_2^2)^{1/2}$ instead of $\delta_1 - \delta_2]$. In a similar way, in the nutating frame, the presence of the audio field reduces the differences of the already reduced Zeeman field ($\omega_e - \omega_m$) in eq 6 even more. This makes the Hamiltonian $\mathcal{H}^{F,I}$ well suited to synchronize the motion of a pair of spins whose resonance frequencies are approximately on opposite sides with respect to the carrier frequency, e.g. $\delta_1 = \delta + \Delta_1$; $\delta_2 = -\delta + \Delta_2$; $\omega_m = (\omega_1^2 + \delta^2)^{1/2}$; $\Delta_1, \Delta_2 \ll \omega_1$; $\Delta_1, \Delta_2 \ll \delta$. For a typical situation in the experiment described below with $\delta = 7.5$ kHz, $\omega_1 = 12.5$ kHz, $\alpha = 0.146$, $\Delta_1 = 600$ Hz, and $\Delta_2 = -300$ Hz, the apparent difference of the effective fields in the nutating frame is only 11 Hz. Therefore, magnetization can be transferred effectively between two homonuclear J-coupled spins, I and S, which are separated by a large frequency offset, provided that the RF carrier is set close to their average resonance frequency. One application of this is the design of an isotropic mixing scheme[11] for the transfer of magnetization between homonuclei with very different chemical shifts, such as C^β and C^γ carbons in aromatic amino acids, or C^α and carbonyl nuclei.

The time evolution for such a two-spin system under the influence of the phase-modulated spin lock field is easily calculated by applying the same manipulations as above to the J-coupling Hamiltonian $\mathcal{H}_J = 2\pi J\ \mathbf{I}\cdot\mathbf{S}$. Usually, $2\pi J$ is much smaller than ω_m and ω_a and after the unitary transforms U_1, U_2, and U_3, $\mathcal{H}_J$ is, to a very good approximation, described by the first-order static Floquet term:

$$\mathcal{H}_J^{F,I} = 2\pi J\{I_zS_z \cos\theta_{IS} + (I_xS_x + I_yS_y)(1 + \cos\theta_{IS})/2\} \quad (7)$$

where $\theta_{IS} = \theta_I - \theta_S$. Only the terms of $\mathcal{H}_J^{F,I}$ which commute with $\mathcal{H}^{F,I}(I) + \mathcal{H}^{F,I}(S)$ (*cf.* eq 6) need to be considered and the solution for the on-resonance case ($\omega_e = \omega_m$) and $\phi = n\pi$ is particularly simple:

$$\mathcal{H}_J^{F,I} = 2\pi J\{I_yS_y(1 + \cos\theta_{IS})/2 + (I_xS_x + I_zS_z)(1 + 3\cos\theta_{IS})/4\} \quad (8)$$

Under such a Hamiltonian, the time evolution of a density matrix $\varrho(t)$, initially in state I_y, is then given by[9]

$$\varrho(t) = I_y(1 + \cos(2\pi J_r t))/2 + S_y(1 - \cos(2\pi J_r t))/2 + (I_zS_x - I_xS_z)\sin(2\pi J_r t) \quad (9)$$

where $J_r = (1 + 3\cos\theta_{IS})J/4$. Thus, after mixing for a time $1/(2J_r)$, I-spin magnetization is fully transferred to S. This mixing therefore is analogous to the original TOCSY and HOHAHA experiments and should be contrasted with "selective homonuclear TOCSY",[12] where the large chemical shift difference beween homonuclei allows the use of heteronuclear Hartmann–Hahn cross polarization schemes to accomplish magnetization transfer. In this latter type of experiment, the minimum time required for transferring magnetization between two coupled spins is $1/J$.

Results and Discussion

Application of the audio-modulated field (*cf.* eq 3) for a duration $\pi[\omega_a(1 + \omega_m/\omega_e)]^{-1}$ corresponds to a 90° rotation about the ϕ axis in the nutating frame. The amplitude of α is preferably adjusted such that the duration of an audio-frequency 90° pulse corresponds to an integer multiple of $2\pi/\omega_m$. It is therefore possible to apply audio-frequency pulse sequences in the nutating frame, in the same manner that complex radio-frequency pulse sequences are applied in the regular rotating frame. In the regular rotating frame, the WALTZ-16 heteronuclear decoupling sequence,[13] which consists of pulse elements that are all integer multiples of 90°, can be used for obtaining reasonably efficient HOHAHA/TOCSY transfer.[14] Exactly the same modulation pattern can also be used in the nutating frame where it considerably improves the behavior of the mixing with respect to frequency offset, second order terms for $\mathcal{H}^F$, and RF inhomogeneity. Figure 1 compares the calculated behavior for a system where magnetization is transferred from a source nucleus (frequency f_s) to a destination nucleus (frequency f_d), coupled with a 50-Hz J coupling, during a mixing period of 13.2-ms duration. Figure 1A represents the transfer amplitude for an unmodulated spin-lock, whereas Figure 1B depicts the AMNESIA-modulated spin-lock with constant phase ϕ, and Figure 1C shows the AMNESIA spin-lock where, in addition, ϕ was modulated according to the WALTZ-16 scheme. Parameters for the RF field were chosen to match those used in the experiment described below for transfer of aromatic β-carbon magnetization into the aromatic ring. For the chosen RF field of strength $\gamma B_1/2\pi = 12.5$ kHz, the AMNESIA scheme is able to transfer magnetization effectively between two spins across a frequency separation of over 20 kHz. For a frequency separation of 15 kHz, corresponding to the separation between C^β and aromatic carbons in phenylalanine residues at 14.1 T, the match condition for this transfer is sufficiently broad that effective transfer can take place for a frequency dispersion of ±0.85 kHz of the source and destination nuclei (Figure 1C). At a 14.1-T field, this frequency range covers the typical Phe-C^β frequency dispersion found in proteins, and that of the aromatic Phe ring carbons, C^γ, C^δ, C^ϵ, and C^ζ, which resonate between 139 and 128 ppm. As can be seen from Figure 1, the match condition in the absence of WALTZ-16 modulation, and particularly in the absence of any audio modulation, is too sharp for transfer from the phenylalanine C^β through the entire aromatic ring up to C^ζ.

The mixing time used for the simulations of Figure 1 is longer than $1/(2J)$ and consequently transfer on the diagonal of this figure ($f_s = f_d$) is past its maximum, which occurs at a mixing time of 10 ms. Although this is not visible at the resolution shown in Figures 1A and 1B, the Hartmann–Hahn match condition broadens for the WALTZ modulated scheme and results in a visible "trench" on the diagonal.

Comparison of Figures 1B and 1C shows that in the case where f_s and f_d are opposite in sign, the maximum transfer with the WALTZ-modulated scheme occurs for smaller values of $|f_s| = |f_d|$ compared to the absence of WALTZ modulation. This is a direct consequence of eq 8 which indicates that the effective

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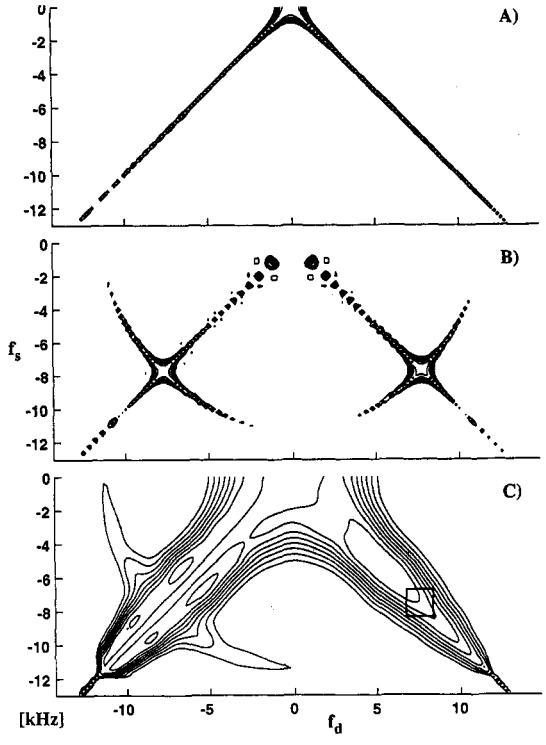

Figure 1. Numerical simulations of the magnetization transfer ($I_y^s \rightarrow I_y^d$) for a system of two spin-$^1/_2$ nuclei where the source spin has frequency f_s and the destination spin has frequency f_d, for a total mixing time of 13.2 ms, a RF field strength $\omega_1/2\pi$ = 12.5 kHz, and J = 50 Hz. Contour levels are at 0.2, 0.3, ..., 0.9. (A) Transfer for a continuous wave RF field, applied along the y-axis; (B) same as (A) in the presence of a constant-phase (y) AMNESIA field (RF along x); (C) same as (B) in the presence of WALTZ-16 audio-modulation along $\pm y$. The amplitude α and frequency $\omega_m/2\pi$ for the AMNESIA modulation are 0.146 and 14.6 kHz, respectively, corresponding to an audio-field strength $\omega_a/2\pi$ of 1.83 kHz. The square drawn in (C) corresponds to f_s = -7.5 ± 0.85 kHz and $f_d = 7.5 \pm 0.85$ kHz. The simulations were carried out as numerical integrations of the respective time-dependent differential equations using the MATLAB® (MathWorks, Inc., Natick, MA) software package.

J coupling decreases when $|f_s - f_d|$ increases. Although eq 8 applies also to the case where no WALTZ modulation is used, the homonuclear Hartmann–Hahn match in the nutating frame is only satisfied when the effective field strength in the rotating frame is closely matched to the audio-modulation frequency of 14.6 kHz. In the WALTZ-modulated case (Figure 1C) the Hartmann–Hahn match is broadened significantly and values with a smaller frequency separation yield more efficient transfer due to their larger effective J coupling, despite their Hartmann–Hahn mismatch in the nutating frame.

The AMNESIA mixing scheme is demonstrated by incorporating it in a pulse sequence designed to correlate $^{13}C^\beta$ with all the aromatic ^{13}C and ^1H frequencies in the phenylalanine side chains (Figure 2). Briefly the pulse scheme functions as follows. After an initial INEPT magnetization transfer[15,16] from H^β to C^β, the frequency of the $^{13}C^\beta$ nuclei is measured in the first semi-constant time[17] interval $t_1^{a,b,c}$ and magnetization is refocused with respect to $^1H^\beta$. The $90°_{\phi 3}$ pulse selects for $^{13}C^\beta{}_y$ magnetization terms. After this pulse the AMNESIA mixing scheme is switched on for a duration of 19.8 ms, corresponding to 1.5 repetitions of the WALTZ-16 supercycle. Then the frequencies of the aromatic ^{13}C resonances are labeled during

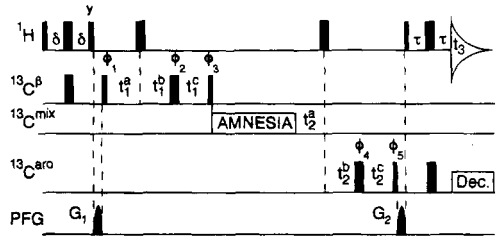

Figure 2. Pulse scheme of the experiment for correlating $^{13}C^\beta$ with the aromatic ring ^{13}C and ^1H signals. Narrow pulses correspond to a flip angle of 90°, wide pulses to 180°. Pulses for which the RF phase is not marked are applied along the x-axis. Carrier frequencies are set to 4.68, 37.8, 87.5, and 130 ppm for the ^1H, $^{13}C^\beta$, $^{13}C^{mix}$, and $^{13}C^{aro}$ pulses, respectively. ^1H pulses are applied at 25 kHz, whereas the ^{13}C 90° and 180° pulses are applied using 3.5 and 7.8 kHz RF field strengths, respectively. The WALTZ-16 modulated AMNESIA field ($\alpha = 0.146$, $\phi = 0$) is applied for a duration of 19.782 ms at a RF field strength of 12.5 kHz. Phase cycling is as follows: $\phi_1 = y$; $\phi_2 = x, y, -x, -y$; $\phi_3 = 4(y), 4(-y)$; $\phi_4 = 8(x), 8(y), 8(-x), 8(-y)$; $\phi_5 = y$; Acq = $4(x, -x), 4(-x, x)$. Pulsed field gradients have a sine-bell shaped amplitude profile with a strength of 25 G/cm at their center. Durations are $G_{1,2}$ = 0.5, 4.5 ms. Quadrature in the t_1 and t_2 domains is obtained by changing the phases ϕ_1 and ϕ_5, respectively, in the usual States-TPPI manner. Delay durations are as follows: $\delta = 1.5$ ms; $\tau = 1.4$ ms. The initial delays for the semi-constant time evolution periods (t_1 and t_2) are set to $t_1^{a,b,c} = 0.85, 0, 0.85$ ms and $t_2^{a,b,c} = 1.4, 0, 1.4$ ms. Increments for those delays are set to $\Delta t_1^{a,b,c} = 81, 60, -21$ μs and $\Delta t_2^{a,b,c} = 100, 13, -87$ μs, resulting in acquisition times of 6.4 (t_1) and 3.2 ms (t_2). If better $^{13}C^\beta$ frequency dispersion is required and a t_1 acquisition time longer than 6.4 ms is needed, it is preferable to insert a frequency selective 180° pulse at C^γ (≈138 ppm) and $^{13}C^\alpha$ (≈58 ppm), centered at a time $(t_1^a + t_1^b - t_1^c)/2$ after the $90_{\phi 1}$ $^{13}C^\beta$ pulse.

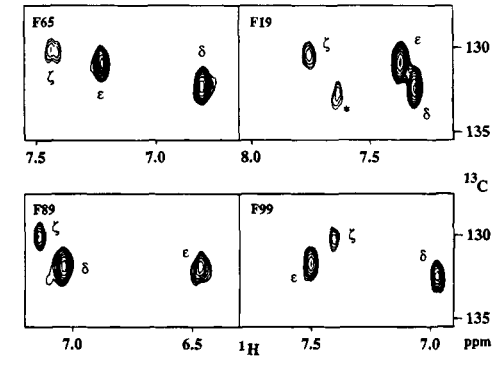

Figure 3. Four (F_2, F_3) cross sections through the 3D $^{13}C-^{13}C-^1H$ AMNESIA spectrum of the calmodulin M13 complex, taken at the F_1 frequencies of the $^{13}C^\beta$ resonances of residues F65, F19, F89, and F99. Each cross section shows the connection to the aromatic δ, ϵ, and ζ ^{13}C and ^1H frequencies of the phenylalanine ring of the respective amino acid. The resonance marked by an asterisk corresponds to the F92 C^δ/H^δ resonance located at an adjacent F_1 frequency.

the second semi-constant time interval $t_2^{a,b,c}$ and magnetization is dephased with respect to the aromatic protons. After a final reverse-INEPT transfer, these aromatic proton signals are detected during the acquisition time, t_3.

Experiments were carried out on a D_2O solution of *Drosophila* calmodulin (CaM), uniformly enriched in ^{15}N and ^{13}C and complexed with a 26 residue natural abundance peptide known as M13. This peptide comprises the binding site of skeletal muscle myosin light chain kinase. The structure of this 20-kDa complex has been established previously by NMR methods.[18] Figure 3 shows four (F_2/F_3) cross sections through the 3D spectrum taken at the $^{13}C^\beta$ frequencies of residues F65, F19,

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F89, and F99. All the connections to the δ, ϵ, and ζ $^{13}C-^{1}H$ frequencies are clearly visible. Except for F12-C^{ζ}, F16-C^{ϵ}, and F16-C^{ζ}, which are subject to partial overlap in the 3D spectrum, the aromatic $^{13}C-^{1}H$ pairs of all 9 Phe residues in CaM could be identified uniquely on the basis of the 3D spectrum, providing a powerful alternative to a recently proposed reverse labeling strategy for obtaining these assignments.[19] The resonance assignments are summarized in Table 1 in the supplementary material.

Nutating frame NMR distinguishes itself from conventional rotating frame NMR by its unique offset dependence. The ability to apply complex pulse sequences in this nutating frame opens new opportunities in a wide range of applications, ranging from protein NMR and solid state magic angle spinning NMR to selective excitation and applications to *in vivo* NMR and magnetic resonance imaging.

Experimental Section

Preparation of the sample of uniformly $^{15}N/^{13}C$-enriched (>95%) calmodulin, complexed with 1 equiv of the synthetic peptide M13, has been described previously.[20] The sample concentration in the present study was 1.0 mM in 99% D_2O, 100 mM KCl, pH 6.3, 35 °C. Experiments were carried out on a Bruker AMX-600 spectrometer, equipped with a wave form generator and a self-shielded z-gradient triple-resonance probehead. The entire modulation pattern for the one and a half WALTZ-16 cycles was programmed as a single shaped pulse (19.8-ms duration). The AMX pulse sequence code is available as supplementary material.

The 3D spectrum results from a $40^{*}(t_1) \times 16^{*}(t_2) \times 384^{*}(t_3)$ data matrix, where n^{*} refers to n complex data points. Total accumulation time was 37.5 h with 128 scans per hypercomplex t_1,t_2-increment. Acquisition times were 6.5 (t_1, C^{β}), 3.2 (t_2, $C^{\delta,\epsilon,\zeta}$), and 49.2 ms ($t_3$, $H^{\delta,\epsilon,\zeta}$). The t_2 time domain was extended by means of forward–backward linear prediction[21] prior to zero filling and Fourier transformation. The size of the absorptive part of the final 3D spectrum was 128 × 256 × 1024.

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Supplementary Material Available: One table containing the resonance assignments of the Phe aromatic protons and carbons and a listing of the AMX-600 pulse sequence of the 3D AMNESIA experiment (5 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm edition of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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